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CHARACTERIZATION OF MELANOMA-ASSOCIATED SURFACE ANTIGENS INVOLVED IN THE ADHESION AND MOTILITY OF HUMAN MELANOMA CELLS

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The functional properties of the melanoma-associated antigens detected by monoclonal antibodies (MAbs) AMF-6 and AMF-7 were investigated. These MAbs were selected previously because of their capacity to block the anti-melanoma reactivity of cytotoxic T-lymphocyte clones AMF-6 and AMF-7 detect a melanoma-associated proteoglycan (MW > 450 - 250 kDa) and a molecular complex, which under reducing conditions consists of 4 compounds of 120, 95, 29 and 25 kDa respectively. AMF-6 reacted strongly with all 30 cultured melanomas and all 41 melanomas in frozen tissue sections. Significant cross-reactivity was only observed with nevi and perineurium, whereas normal skin melanocytes were negative. AMF-7 reacted with all 25 cultured melanomas and all 34 melanomas in frozen sections. AMF-7 cross-reacted with a proportion of nevi and endothelial cells from small vessels. The antigen detected by AMF-6 and AMF-7 could not be modulated by retinoic acid or recombinant γ -IFN, which induced or enhanced the expression of HLA-DR, HLA-DQ and Class-I MHC antigens. In addition, the antigens were not readily modulated when cells were incubated in excess amounts of AMF-6 and AMF-7. Interestingly, the antigen detected by AMF-7 was strongly associated with the adhesion and cytoplasmic spreading of melanoma cells to plastic surfaces and monolayers of vascular endothelial cells. AMF-6 did not block the adhesion of melanoma cells but delayed cytoplasmic spreading. Both AMF-6 and AMF-7 blocked fibronectin-induced chemotactic motility and chemokinesis of melanoma cells. In addition to their membrane localization, the antigens detected by AMF-6 and AMF-7 were also abundant in extracellular adhesion plaques deposited by cultured melanoma cells.

Our results indicate that the high-MW melanoma-associated proteoglycan and the antigen detected by AMF-7 are associated with adhesion and/or cytoplasmic spreading and motility of human melanoma cells, suggesting that these antigens are associated with the (hematogenic) dissemination of human melanoma. This is supported by the finding that AMF-7 stained primary tumors heterogeneously, whereas metastases were homogeneously stained.

Recently, MAbs reacting with antigens on murine and human melanoma cells that are associated with the adhesion and motility of these cells have been described (Vollmers and Birchmeier, 1983a,b; Dippold *et al.*, 1984; Cheresch *et al.*, 1984). Both cell-cell adhesion and cell motility are important factors in the process of metastasis (reviewed by Fidler and Nicolson, 1980; and Nicolson, 1982, 1984).

One critical step in hematogenic metastasis is that blood-borne tumor cells are arrested in the microcirculation by attachment to vascular endothelium (Kramer and Nicolson, 1979; Kramer *et al.*, 1980; Terranova *et al.*, 1983). After this adhesion step, extravasation occurs and the tumor cells invade the un-

derlying basement membrane and tissues (Kramer *et al.*, 1980; Nicolson, 1982). Tumor cells probably utilize several mechanisms to penetrate normal tissue (Nicolson, 1982), but active tumor-cell motility also contributes to the invasive process (Poste and Fidler, 1980; Haemmerli *et al.*, 1982; Volk *et al.*, 1984). Therefore MAbs that block adhesion, motility and migration of tumor cells may detect antigens which are associated with the hematogenic dissemination of these tumors. Indeed, Vollmers and Birchmeier (1983a,b) observed that MAbs against B16 mouse melanoma, which were selected for their capacity to block the adhesion of B16 cells, also inhibited the formation of lung metastasis *in vivo*. These anti-B16 MAbs were not specific. Cross-reactivity was observed with a large variety of malignant and virally transformed cells of both murine and human origin (including melanoma), but not with non-transformed cells (Vollmers and Birchmeier, 1983b).

Other MAbs that detect antigens associated with adhesion of human melanoma cells include MAb R24 which reacts with the GD3 ganglioside present on melanomas, melanocytes, astrocytomas, astrocytes and subsets of sarcomas. R24 causes melanoma cells to aggregate and to detach from plastic surfaces (Dippold *et al.*, 1984). GD3 gangliosides can be considered as cellular adhesion molecules, since this molecule is present in the extracellular adhesion plaques deposited by human melanoma cells at the interface between the tumor cells and their substrate (Cheresch *et al.*, 1984).

In the present study we describe 2 MAbs, AMF-7 and PAF-1, which detect the same melanoma-associated surface antigen. This antigen is associated with adhesion, spreading and motility of human melanoma cells. The last two properties were also blocked by another MAb (AMF-6) which detects a melanoma-associated high-molecular-weight proteoglycan. These results indicate that the antigens detected by these MAbs may be associated with metastasis.

This notion is supported by the *in vivo* distribution of the antigen detected by AMF-7. Primary tumors stained heterogeneously contained AMF-7-negative cells, whereas metastases were stained homogeneously.

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MATERIAL AND METHODS

Cells and cell lines

Melanoma cells were derived from both short-term cultures and long-term cell lines established in our laboratory (De Vries *et al.*, 1972). The short-term-cultured (4 weeks in culture, 6th passage) BK melanoma cells used to immunize mice for MAb production were obtained from a cervical lymph-node metastasis of a 44-year-old male patient. All melanoma cells and the other adherent cell cultures derived from normal and non-melanoma tumor tissue were cultured in Dulbecco's modification of Eagle's minimal essential medium (DMMEM) supplemented with streptomycin (50 $\mu\text{g}/\text{ml}$), penicillin (100 IU/ml), NaH_2CO_3 (2 g/l), glutamine (2 mM) and 10% FCS (GIBCO, Glasgow, Scotland). The mammary carcinoma cell cultures used in this study were also supplemented with insulin (5 $\mu\text{g}/\text{ml}$). The EBV-transformed B-cell lines, the T leukemic cell lines and the myeloid leukemia cell lines were cultured in serum-free medium (Yssel *et al.*, 1984a). Normal T lymphoblasts were grown in serum-free medium in the presence of interleukin-2 and an irradiated feeder cell mixture (Spits *et al.*, 1982a,b). Vascular endothelium cell cultures were derived from human umbilical veins (Jaffe *et al.*, 1973). The cells were cultured in Iscove's medium supplemented with 10% human serum, 3.3×10^{-5} M isobutyl methylxanthine and 5×10^{-4} M dibutyryl cyclic AMP.

Production and selection of MAbs against melanoma

BALB/c mice were immunized with BK melanoma cells (Spits *et al.*, 1984b). Hybridomas reacting with melanoma cell surface antigens and not with autologous T lymphoblasts were selected for further testing and finally screened for their capacity to inhibit the cytotoxic reactivity of a CTL clone against autologous BK melanoma cells. The hybridomas producing these antibodies were cloned and subcloned at concentrations of 0.5 cells/well in 96-well microtiter plates and injected into pristane (2,6,10,14 tetramethyl pentadecane)-treated BALB/c mice for ascites production. Two MAbs, AMF-6 (IgG1) and AMF-7 (IgG1), which inhibit the cytotoxic effects of CTL clones with anti-melanoma reactivity (data not shown) were selected for investigation of their effects on the biological functions of human melanoma cells. AMF-6 was shown to precipitate a molecular complex consisting of 2 bands with MW of more than 450 and 250 kDa, respectively, like the melanoma-associated proteoglycan described by others (Natali *et al.*, 1981; Bumol and Reisfeld, 1982). AMF-7 reacts under non-reducing conditions with a molecular complex consisting of 2 bands having MWs of 150 and 90 kDa, respectively. Under reducing conditions the antigen detected by AMF-7 consists of 4 subunits with MWs of 120, 95, 29 and 25 kDa (data not shown). PAF-1 is an MAb raised against short-term cultured BK melanoma cells on a different occasion. The selection protocol was similar to that described above for AMF-7, except that the last selection step comprised a screening of the supernatants for their capacity to prevent adhesion of melanoma cells to plastic surfaces. Immune precipitation showed that PAF-1 detected the same 150- to 95-kDa antigen as did AMF-7 (data not shown).

Monoclonal antibodies

The MAb W6/32 which reacts with a framework determinant on HLA-A, B and C was obtained from Sera-Lab, Crawley Down, UK. Q 5/13 directed against HLA-DR (Quaranta *et al.*, 1980) was kindly provided by Dr. S. Ferrone (Valhalla, NY). SPV-L3, which reacts with HLA-DQ, was produced in our laboratory (Spits *et al.*, 1984a). AMF-2 was raised in our laboratory and reacts with a 22-kDa surface antigen expressed on melanoma and a variety of other cells including neuroblastomas, other non-melanoma tumors, EBV-transformed B-cell lines and normal tissues. AMF-8 (IgG1) was raised in our laboratory and also reacts with a melanoma surface antigen whose MW has not yet been determined. As far as tested, AMF-8 is not melanoma-restricted and it cross-reacted with EBV-transformed B-cell lines and myeloid leukemia cell lines. PA-LM1 (IgG1) and PA-LM2 (IgG1) were obtained from Dr. D. Ruiter (Leiden, The Netherlands). Both antibodies react with melanoma-associated antigens (Ruiter *et al.*, 1985). PA-LM2 detects an antigen consisting of 2 subunits with estimated MWs of 100 and 95 kDa, which is preferentially expressed on melanomas and not on nevi (Ruiter *et al.*, 1985). The MW of the antigen detected by PA-LM1 has not yet been determined. The MAb 96.5, which reacts with the melanoma-associated p97 antigen (Hellström *et al.*, 1982), was kindly provided by Drs. I. and K.E. Hellström (Seattle, WA). OKT-9, which is directed against the transferrin receptor (Trowbridge and Omary, 1981) was purchased from Ortho, Raritan, NJ.

Membrane immunofluorescence

Membrane immunofluorescence on cultured cells and cell lines was carried out as described previously (Yssel *et al.*, 1984b). Adherent cells were detached with EDTA, resuspended in DMEM + 10% FCS, and incubated for 1 hr with occasional shaking. After washing with PBS containing 0.5% BSA and azide (0.1%), 5×10^5 cells were incubated for 20 min at 0°C with the MAb at a dilution of ascites of 1:2,000 to 1:5,000. After this incubation period the cells were washed 3 times with PBS (0.5% BSA and azide) and incubated with FITC-labelled GAM-Ig (1:20 diluted, Nordic, Tilburg, The Netherlands) for 20 min at 0°C. Subsequently, the cells were washed 3 more times with PBS and analyzed with a FACS (FACS IV, Becton-Dickinson, Mountain View, CA).

Detection of extracellular matrix by immunofluorescence

For analysis of the extracellular matrix, melanoma cells were cultured on glass coverslips for 24-48 hr. Melanoma cells were carefully detached with EDTA and their removal was checked microscopically. Coverslips were incubated with MAbs, then washed, stained with GAM-Ig FITC and examined with a fluorescence microscope.

Immunoperoxidase staining of frozen tissue sections

For immunoperoxidase staining of frozen sections (Ruiter *et al.*, 1985), frozen sections (4 μ) were air-dried and fixed in acetone for 10 min. MAbs were added at an appropriate dilution and incubated for 60 min at room temperature. The sections were washed

TABLE I - DISTRIBUTION OF THE MELANOMA-ASSOCIATED ANTIGENS DETECTED BY MABs AMF-6 AND AMF-7

MAB	Melanoma				Non-melanoma tumor		Normal cells/tissues	
	Primary		Metastases		Membrane IF	Frozen sections	Membrane IF	Frozen sections
	Membrane IF	Frozen sections	Membrane IF	Frozen sections				
AMF-6	2/2	6/6	28/28	35/35	0/22 ¹	0/12 ²	0/22 ³	2/71 ⁴
AMF-7	2/2	5/5	15/15	29/29	0/21 ⁵	0/12 ²	0/15 ⁶	3/55 ⁷

¹Including: 3 neuroblastomas, 2 mammary carcinomas, 2 oat-cell carcinomas, 1 bladder carcinoma, 1 teratocarcinoma, 1 cervix carcinoma, 3 myeloid leukemias, 1 erythroid leukemia, 3 T-cell leukemias, 2 Burkitt lymphomas, 2 B lymphomas, and B-16 murine melanoma. ²Including: 2 ovarian carcinomas, 2 colon carcinomas, 2 lung carcinomas, 1 neurinoma, 1 thyroid carcinoma, 2 neuroblastomas and 2 mammary carcinomas. ³Including: 5 EBV-transformed B-cell lines, 6 T lymphoblast, 3 normal skin fibroblast, 1 normal liver cell, 1 normal bladder cell, 2 vascular endothelial cell, 3 monocyte, 1 melanocyte culture(s). ⁴Positive reactions were observed with 5/6 naevi, 2/2 perineurium. ⁵As under ¹ except that AMF-7 was only tested on one B lymphoma. ⁶Similar to ³ except that AMF-7 was tested on 3 EBV-transformed B-cell lines, 3 T lymphoblast and 2 normal skin fibroblast cultures. ⁷Positive reactions were observed with endothelial cells of small vessels, 8/17 nevi and weak reactivity with 4/5 hair follicles.

in phosphate-buffered saline (pH 7.4) and subsequently incubated with rabbit anti-mouse immunoglobulin-horseradish peroxidase conjugate (Dako, Copenhagen, Denmark). Tissue sections were washed and stained by incubation in acetate-buffered (pH 5.0) 3-amino-9-ethylcarbazole (Aldrich, Milwaukee, WI), dimethyl formamide and hydrogen peroxide. After washing with acetate buffer, sections were counterstained in Mayer's hematoxylin and mounted in Aquamount (Gurr, B.D.H., Poole, UK).

Adhesion of melanoma cells to plastic surfaces and monolayers of vascular endothelial cells

The adhesion of melanoma cells to plastic surfaces or monolayers of vascular endothelial cells was measured in an ELISA. MAb SPV-L8, which detects a framework determinant on HLA-B and which reacts with all cultured melanoma cells tested, was directly labelled with horseradish peroxidase (HRPO, Sigma, type VI). Before labelling with HRPO, mouse SPV-L8 ascites was purified by ammonium sulfate precipitation. Melanoma cells were detached with EDTA, washed with PBS and allowed to recover in DMEM + 10% FCS and 20 mM HEPES for 1 hr at 37°C with occasional shaking. Melanoma cells 5×10^6 were incubated with 150 μ l HRPO-labelled SPV-L8 at a dilution of 1:50 for 45 min at 0°C. After 3 washings, 100 μ l culture medium containing 5×10^3 melanoma cells were seeded in 0.2-ml wells (Costar, 3596, Cambridge, MA) in the presence or absence of the MABs to be tested. Unless stated otherwise, the MABs were tested at dilutions of 1:200 of ascites. In our standard assay, melanoma cells were allowed to adhere for 4 hr at 37°C and 5% CO₂. In the absence of MABs the majority (60–80%) of the cells of melanoma cultures adhered to plastic during this incubation period. Some melanoma cultures (MEWO, KEU and GEVA) required longer incubation times (up to 10 hr) before the majority of cells attached. After 4 hr incubation in general, approximately 10–20% of melanoma cells attached. After the 4-hr incubation period, the wells were gently rinsed twice with warm (37°C) PBS + 0.2% BSA to remove non-adherent cells. The adherent cells were stained with 100 μ l containing 10^{-2} ng TMB (3,3',5,5'-tetramethylbenzidine, crystalline, Sigma, St. Louis, MO). The reaction was stopped after 5–60 min incubation (depending on the intensity of the color of the substrate) by adding 100 μ l 0.8 M H₂SO₄. The intensity of the color was measured in a multiscan photo-spectrometer (Titertek, Skatron, Lier, Norway)

at a wavelength of 450 nm. Wells in which no cells were seeded served as controls. The staining correlated directly with the number of adherent cells as determined by a standard calibration curve (not shown). The value obtained with cells incubated in the absence of MABs was set at 100%. Adhesion of melanoma cells to vascular endothelial cells was carried out similarly in microtiter wells containing confluent monolayers of endothelial cells.

Migration and motility of melanoma cells

The motility of melanoma cells was measured in a 48-well micro-chemotaxis assembly (NeuroProbe, Bethesda, MD). The bottom wells were filled with 25 μ l of medium with or without human plasma-derived fibronectin (300 μ g/ml, Armour, Kankakee, IL) which was used as a chemo-attractant (Lacovara *et al.*, 1984). Subsequently, 10- μ m thick, 8- μ m pore diameter polycarbonate membranes without PVP coating (Nucleopore, Pleasanton, CA) were fixed in place to separate bottom from top wells, and 2.5×10^3 melanoma cells in 50 μ l medium containing 2% human serum were added to the top wells in the presence or absence of MABs. Melanoma cells were detached with EDTA and allowed to recover by incubating in medium for 1 hr at 37°C, 5% CO₂ with occasional shaking. Random migration (bottom and top wells containing medium only), chemokinesis (bottom and top wells both containing medium with fibronectin), and chemotaxis (only bottom wells containing medium with fibronectin) were determined after 2–6 hr incubation at 37°C and 5% CO₂. The number of cells that migrated through the filter and adhered to the underside of the filter were quantified after the cells on top of the filter were wiped off. The filter was air-dried and the cells were stained with Coomassie blue and counted microscopically.

RESULTS

Distribution of the antigens detected by AMF-6 and AMF-7

Membrane immunofluorescence studies showed that AMF-6 (IgG1) and AMF-7 (IgG1) reacted strongly with 30/30 and 25/25 melanoma cells respectively, both derived from cell lines and short-term cultures. No reactivity was observed with cell cultures derived from non-melanoma tumor or normal tissues (including 3 neuroblastomas which, like melanomas, are of neuroectodermal origin) and cultured vascular endothelial cells derived from umbilical veins (Table I). In

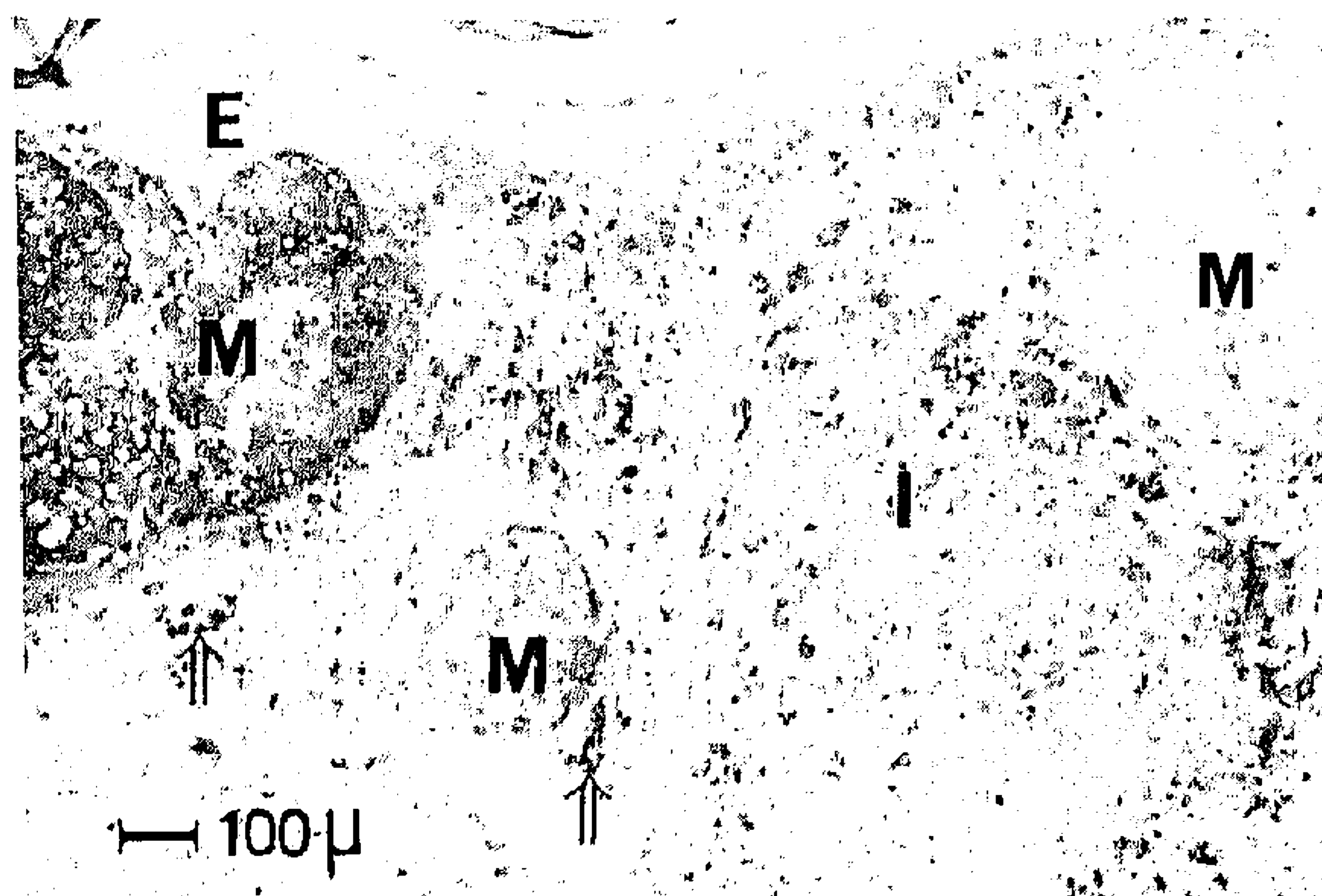


FIGURE 1 – Primary skin melanoma stained with AMF-7 showing marked variation in staining intensity between different melanoma nodules (M). Endothelial cells of adjacent capillaries also stain (arrows). E = epidermis, I = inflammatory infiltrate. Hematoxylin counterstaining.



FIGURE 2 – Melanoma metastasis showing marked diffuse staining with AMF-7. Endothelial cells also stain (arrows). Hematoxylin counterstaining.

addition, AMF-7 failed to stain murine B16 melanoma cells. AMF-6 and AMF-7 also reacted with 41/41 and 34/34 melanomas in immune-peroxidase-stained frozen tissue sections. AMF-7 showed significant cross-reactivity with endothelial cells of very small vessels (Fig. 1), but not with those of large vessels, and with a proportion of the nevi. AMF-6 also reacted with perineurium and most nevi, and showed weak reactivity with hair follicles. Normal skin melanocytes were negative. A striking observation was that AMF-7 stained primary tumors heterogeneously. Sometimes considerable percentages of negatively and very weakly stained cells were observed (Fig. 1), whereas metastasis stained homogeneously (Fig. 2).

Modulation of the antigen detected by AMF-7

In order to investigate whether the antigen could be modulated, melanoma cells were incubated with recombinant γ -IFN (kindly provided by Dr. H. Schellekens, TNO, Rijswijk, The Netherlands) or retinoic acid. Incubation for 48 hr with γ -IFN (500 U/ml) or retinoic acid (10^{-6} M) had no effect on the expression of AMF-7. Similarly, the expression of antigens detected by AMF-2, AMF-6 and AMF-8, and the transferrin receptor on the melanoma cells as detected by OKT-9 were not affected, even after incubation periods of up

to 6 days (not shown). In contrast, in all melanoma cultures, a strong induction or increase in the expression of HLA-DR was observed after 24-48 hr incubation with 500 U γ -IFN (Table II). DQ antigens as detected by MAb SPV-L3 were absent from melanoma cells but incubation with γ -IFN induced the expression of HLA-DQ in 6/9 melanomas. Retinoic acid induced DR expression but had no effect on the expression of HLA-DQ. This indicates not only that HLA-DR and HLA-DQ on melanoma cells are differently expressed, but that their expression is also regulated differently. The expression of Class-I MHC antigens was already high, but both γ -IFN and retinoic acid generally induced an increase in the expression of these antigens (Tables II and III). Cultivation of melanoma cells in the presence of an excess of AMF-6 or AMF-7 for periods up to 6 hr did not result in modulation of the antigen, although in some melanoma cultures cap and patch formation was observed (not shown).

The effects of AMF-6 and AMF-7 on adhesion of melanoma cells to plastic surfaces and monolayers of vascular endothelial cells

The effects of AMF-6 and AMF-7 on adhesion of various melanoma cells derived from short-term cultures and cell lines was tested with an ELISA. AMF-7

TABLE II – EFFECT OF RECOMBINANT γ -IFN ON EXPRESSION OF THE ANTIGEN DETECTED BY AMF-7 AND CLASS-I AND CLASS-II MHC ANTIGENS

Melanoma culture ¹	AMF-7 Channel No.	HLA-DR with maximal	HLA-DQ fluorescence	HLA-A,B,C intensity ²
L-2	130 (–2) ³	174 (25)	65 (33)	214 (–12)
BK	80 (–8)	165 (136)	69 (46)	181 (20)
Hor	86 (6)	127 (100)	27 (–5)	221 (18)
Keu	150 (–8)	171 (143)	125 (94)	220 (18)
NKI-4	137 (–7)	168 (113)	69 (28)	198 (–14)
NK1-1	157 (–4)	196 (133)	118 (71)	229 (43)
MeWo	81 (4)	64 (16)	53 (5)	202 (34)
O-mel	117 (–7)	122 (17)	22 (–5)	NT
Ba-H	99 (–12)	116 (42)	51 (27)	196 (25)

¹Melanoma cells were incubated with 500 IU/ml γ -IFN (optimal concentration) for 24 hr. ²Fluorescence intensity expressed as the channel number representing maximal fluorescence intensity. Channel numbers are plotted on a logarithmic scale. ³In parentheses: difference in channel number with melanoma cells cultured in the absence of γ -IFN. Control values of melanoma cells tested in the absence of MABs varied between channel numbers 21 and 42.

TABLE III - THE EFFECT OF RETINOIC ACID (10^{-6} M) ON THE EXPRESSION OF ANTIGENS DETECTED BY AMF-7 AND CLASS-I AND CLASS-II MHC ANTIGENS

Melanoma culture ¹	AMF-7 Channel No.	HLA-DR with maximal	HLA-DQ fluorescence	HLA-A,B,C intensity ²
L-2	125 (-7) ³	147 (-12)	35 (3)	210 (-16)
BK	84 (-8)	122 (93)	24 (1)	185 (24)
Hor	73 (-7)	88 (67)	24 (-8)	211 (8)
NKI-4	131 (-13)	150 (6)	36 (-5)	205 (-7)

¹Melanoma cells were incubated with 10^{-6} M retinoic acid (optimal concentration) for 24 hr. ²Fluorescence intensity is expressed as channel number representing maximal fluorescence intensity. Channel numbers are plotted on a logarithmic scale. ³In parentheses: difference in channel number with melanoma cells cultured in the absence of retinoic acid. Control values of melanoma cells tested in the absence of MAb varied between channel numbers 21 and 35.

inhibited the adhesion of all 14 melanoma cell cultures tested to both plastic surfaces and monolayers of vascular endothelial cells (Table IV). Average inhibition was 59% (39-79%). In contrast, AMF-2, AMF-8, PA-LM1 and the MAbs directed against HLA-DR(Q5/13), HLA-DQ (SPV-L3) and HLA-A,B,C (W6/32) had no effect. Moreover, AMF-6, which is directed against a melanoma-associated sulfated proteoglycan, had no significant effect, although cytoplasmic spreading was somewhat inhibited. PA-LM2, which was selected because it stains melanoma cells in frozen tissue sections and which reacted with an antigen consisting of 2 bands in immunoblots with estimated MWs of 100 and 95 kDa respectively (Ruiter *et al.*, 1985), also inhibited the adhesion of melanoma cells. PA-LM2 did not affect the adhesion of 2/14 melanoma cultures. However, immunofluorescence studies showed that these 2 melanomas failed to react with PA-LM2. This is in line with data obtained by Ruiter *et al.* (1985) who showed that PA-LM2 did not stain all melanomas in frozen sections. PAF-1, which was selected for its capacity to inhibit the adhesion of melanoma cells, and which reacted with the same antigen as detected by AMF-7, also inhibited the adhesion of cells from all 14 melanomas tested. This selection procedure is thus shown to be very effective in obtaining MAbs directed against cellular adhesion molecules. AMF-7 and PAF-1 also inhibited the adhesion of melanoma cells derived from 4 different cultures to monolayers of vascular endothelial cells. Average inhibition was somewhat lower (51%) (32-65%) than that observed in adhesion to plastic surfaces. Finally, adhesion of cultured skin fibroblasts, which do not express the antigen(s) detected by AMF-7, PA-LM2 and PAF-1, was not inhibited by these MAbs. In addition to its adhesion-inhibitory effects, AMF-7 also delayed cytoplasmic spreading of the cells which did adhere. After 4 hr

incubation, a relatively large proportion (>25%) of the adherent cells were still rounded up and not spread out. A similar but less marked delay in cytoplasmic spreading was also observed with AMF-6 (data not shown).

Correlation between the expression of the antigen detected by AMF-7 and adhesion

In the above adhesion experiments, various melanoma cultures varied in their degree of adhesion. The majority of melanoma cells adhered efficiently during the 4 hr test period and in general 70-80% of the cells were attached and spread out (Table V). Some cell lines, like MEWO and GEVA, however, adhered very poorly. After 4 hr incubation, only 10-20% of the cells attached and it took 10 hr before 70-80% of cells adhered. In order to determine whether there was a relation between the degree of expression of the antigen reacting with AMF-7 and the capacity to adhere to plastic surfaces, both parameters were evaluated. Table V shows that there is no correlation between the quantity of the antigen expressed on the cell surface, as determined by FACS analysis, and the capacity of the cells to adhere. The GEVA cells, which adhere very poorly, expressed abundant antigen detected by AMF-7 (Table V). In contrast, the short-term melanoma culture B-K, which adhered very efficiently, expressed relatively low quantities of the antigen. This indicates that although the antigen detected by AMF-7 is strongly associated with cell-substrate and cell-cell adhesion, other melanoma surface antigens with adhesion properties also contribute to the adhesion of melanoma cells.

Analysis of extracellular adhesion plaques deposited by melanoma cells

Melanoma cells were grown on glass coverslips for 24-48 hr and analyzed for the presence of adhesion

TABLE IV - EFFECTS OF MAbs AGAINST MELANOMA SURFACE DETERMINANTS AND CLASS-I AND CLASS-II MHC ANTIGENS ON THE ADHESION OF MELANOMA CELLS TO PLASTIC OR ENDOTHELIAL CELLS

MAb antibody added	% Inhibition of adhesion		
	Plastic		Endothelial cells
	Melanoma cells (mean \pm SD 14 melanomas)	Fibroblasts	Melanoma cells (mean \pm SD 5 melanomas)
AMF-2	12 \pm 3	4	14 \pm 3
AMF-6	2 \pm 4	-5	4 \pm 2
AMF-7	59 \pm 5	9	51 \pm 6
PA-LM1	1 \pm 5	NT	2 \pm 2
PA-LM2	40 \pm 14	NT	42 \pm 9
PAF-1	50 \pm 4	-8	45 \pm 5
Q 5/13	-4 \pm 3	-8	-2 \pm 3
W6/32	3 \pm 4	2	3 \pm 3

TABLE V - EXPRESSION OF ANTIGEN DETECTED BY AMF-7, ADHESION AND MORPHOLOGY OF THE MELANOMA CELLS

Melanoma culture	Expression ¹ AMF-7	% Cells attached		Morphology
		4 h	10 h	
O-mel	124	45	> 80	Spindle-shaped
Ba-H	111	70	NT	Fibroblast-like
DO	65	75	NT	Epithelioid
GEVA	158	15	70	Spindle-shaped
Keu	154	15	65	Fibroblast-like
BK	75	80	NT	Epithelioid
MeWo	72	5-10	70	Spindle/epithelioid
NKI-4	144	80	NT	Epithelioid

¹Fluorescence intensity expressed as the channel number representing maximal fluorescence intensity. Channel numbers are plotted on a logarithmic scale.

plaques. The high-MW proteoglycan, as detected by AMF-6 and the antigen detected by AMF-2, were abundant in the adhesion plaques that were deposited predominantly along cell membranes (Fig. 3a,b). AMF-7 was also equally distributed in the extracellular adhesion plaques (Fig. 3c). In contrast, Class-I and -II MHC antigens and the antigen detected by 96.5 were absent (not shown). This indicates that membrane surface antigens detected by AMF-2, AMF-6 and AMF-7 are also secreted in the extracellular adhesion plaques, illustrating that these molecules are also components of the extracellular matrix.

The effects of MAbs on the chemotactic motility of melanoma cells

Adhesion of migrating tumor cells to vascular endothelium is an important step in metastasis (Kramer and Nicolson, 1979; Kramer *et al.*, 1980; Terranova *et al.*, 1983). After the cells adhere, they invade the endothelial cell layer, the underlying basement membrane and tissues. This migration of melanoma cells may be a unidirectional mode of locomotion along a path of preferential adhesion (Carter, 1965). It may occur through gradients of fibronectin (Birdwell *et al.*, 1978; Kramer *et al.*, 1980). High concentrations of fibronectin are present in the sub-epithelial, basal laminal part of the endothelial lining of blood vessel walls, whereas low levels are found on the apical surfaces of endothelial cells (Birdwell *et al.*, 1978; Kramer *et al.*, 1980). Therefore, we investigated the effect of AMF-7 and other MAbs on motility and migration of NKI-4 melanoma cells induced by fibronectin. Preliminary data show that fibronectin-induced chemotaxis and chemokinesis are strongly inhibited by AMF-7 (Table VI). MAbs AMF-6 and, to a lesser extent W6/32 (anti-class-I HLA), also exerted inhibiting effects. Q 5/13 (anti-HLA-DR) and AMF-8 were ineffective. Only 4-7% of the NKI-4 cells migrated through the filter under the influence of a fibronectin gradient (chemotaxis) whereas this percentage varied between 1 and 2% for chemokinesis. Spontaneous migration was negligible (less than 20 cells passed the filter). This indicates that the antigen detected by AMF-7, designated melanoma-associated cellular adhesion molecule-1 (MACAM-1) is not only associated with adhesion of melanoma cells to vascular endothelium but also plays a role in the motility and migration of these cells. The high-MW proteoglycan does not significantly affect adhesion but blocks chemotactic and chemokinetic motility of melanoma cells.

DISCUSSION

We describe here MAbs that react with melanoma cell-surface antigens associated with biological functions of human melanoma cells. MAbs AMF-7 and PAF-1 detect a melanoma-associated cellular adhesion molecule designated MACAM-1. MAb AMF-6 precipitates a molecular complex consisting of 2 bands with MWs of >450 and 250 kDa, similar to the sulfated proteoglycan described by Natali *et al.* (1981) and Bumol and Reisfeld (1982). AMF-6 and AMF-7 were initially selected by virtue of their ability to inhibit the anti-melanoma reactivity of cytotoxic T-cell clones isolated from mixed lymphocyte cultures and mixed lymphocyte melanoma cell cultures (De Vries and Spits, 1984). PAF-1 was selected because it inhibits adhesion of melanoma cells to plastic surfaces. AMF-6 and AMF-7 react as far as tested with all melanomas both in tissue culture and in frozen sections. AMF-7 only showed significant cross-reactivity with a certain proportion of nevi and endothelial cells of small vessels, whereas endothelial cells of larger vessels and cultured vascular endothelial cells derived from umbilical veins were negative. The antigen detected by AMF-7 is a complex molecule. Under non-reducing conditions, it consists of 2 components with MWs of 150 and 90 kDa. In a reduced state the molecular complex consisted of 4 moieties with MWs of 120, 45, 29 and 25 kDa (data not shown). An antigen similar to MACAM-1 has been described by Mitchell *et al.* (1980). AMF-6 was found to cross-react with most nevi and with perineurium. The antigen detected by AMF-7 is trypsin-resistant whereas the melanoma-associated proteoglycan is trypsin-sensitive (data not shown). Neither antigen could not be modulated by γ -IFN or by retinoic acid. In contrast, these immune modulators had differential effects on induction or enhancement of expression of HLA-DR and HLA-DQ. Expression of HLA-DR was generally enhanced by γ -IFN but expression of HLA-DQ could not be induced in 3/9 melanomas (HOR, MEWO and O-mel). Retinoic acid significantly enhanced HLA-DR expression in 3/4 melanomas, but did not induce HLA-DQ. This suggests that the expression of HLA-DR and HLA-DQ on melanoma cells is independently regulated. Although in some melanomas cap formation was observed, neither MACAM-1 nor proteoglycan were readily modulated by incubation with an excess of antibody. Patch formation was generally observed after prolonged culture in the presence of AMF-6 and AMF-7. This indicates that these antigens are laterally mo-

TABLE VI - INHIBITION OF FIBRONECTIN-INDUCED CHEMOTAXIS AND CHEMOKINESIS BY MABs DIRECTED AGAINST MELANOMA SURFACE ANTIGENS AND CLASS-I AND CLASS-II MHC ANTIGENS ON NKI-4 CELLS

MAb ¹	% Inhibition (mean \pm SD 3 experiments)	
	Chemotaxis	Chemokinesis
AMF-6	52 \pm 8	47 \pm 7
AMF-7	90 \pm 3	95 \pm 2
AMF-8	5 \pm 2	15 \pm 5
W6/32	28 \pm 4	34 \pm 4
Q5/13	9 \pm 2	14 \pm 5

¹MABs were added at a concentration of 10 μ g/ml of purified antibody.

tile. Similar patch-formation has been reported for a large variety of integral membrane proteins (Nicolson, 1976). Whether MACAM-1 and proteoglycan are indeed integral membrane constituents is presently being investigated.

Interestingly, we found that MACAM-1 was associated with the adhesion (and cytoplasmic spreading) of melanoma cells to plastic surfaces and monolayers of vascular endothelial cells obtained from umbilical veins. AMF-6 only delayed cytoplasmic spreading (as reported by Reisfeld *et al.*, 1984), but did not significantly affect adhesion.

In a 4-hr assay AMF-7 blocked adhesion of approximately 60% of melanoma cells to plastic and 50% of these cells to endothelial cells. Adhesion of migrating tumor cells to vascular endothelium is an important step in hematogenic metastasis (Fidler and Nicolson, 1980; Nicolson, 1982, 1984) followed by extravasation and invasion of endothelium and underlying basement membrane and tissues (Kramer and Nicolson, 1979; Kramer *et al.*, 1980; Terranova *et al.*, 1983). Although tumor cells probably utilize several mechanisms to penetrate normal tissue during invasion (Nicolson, 1982), active cell motility contributes also to the invasive process (Poste and Fidler, 1980; Haemmerli, 1982; Volk *et al.*, 1984). Preliminary experiments showed that AMF-6 and AMF-7 inhibited the fibronectin-induced chemotactic and chemokinetic motility of melanoma cells, indicating that both antigens are associated with migration of melanoma cells. Experimental systems have shown that MABs which block adhesion of tumor cells *in vitro* also inhibit metastasis *in vivo* (Vollkers and Birchmeier, 1983a) and that MACAM-1 and proteoglycan are associated with migration of melanoma cells; it is therefore tempting to speculate that these antigens play a role in hematogenic dissemination of human melanoma. This is supported by the distribution of MACAM-1 in frozen tissue sections. Primary melanoma tumors are stained heterogeneously and contain a considerable number of AMF-7-negative melanoma cells. In contrast, microsatellites and distant metastases stained homogeneously in all instances.

MAB/PAF-1, which was selected by screening hybridoma supernatants for their capacity to inhibit adhesion of melanoma cells to plastic, detected the same antigen as AMF-7, indicating that this screening

FIGURE 3 - Adhesion plaques deposited by melanoma cells (L-2) on glass cover-slips. Staining with AMF-2 (a), AMF-6 (b) and AMF-7 (c).

method is effective for obtaining MAbs reacting with MACAMs. This has already been reported for experimental systems by Vollkers and Birchmeier (1983a,b) who showed that MAbs against murine B16 melanoma, selected for their capacity to block adhesion of B16 melanoma cells to plastic, were also effective in reducing experimental lung metastasis *in vivo*. In contrast to AMF-7 which reacts preferentially with melanoma cells, these anti-B16 antibodies, however, cross-reacted with a large variety of other malignant and virally transformed cells including human melanoma. Adhesion was also inhibited by MAb PA-LM2 which detects a melanoma-associated antigen consisting of 2 components with MWs of 100 and 95 kDa. PA-LM2 probably detects an antigen other than MACAM-1 since it does not react with all melanomas (Ruiter *et al.*, 1985). Inhibition of adhesion observed with AMF-7, PA-LM1 and PAF-1 was never complete. Maximal inhibitions of 80% (with an average of 60%) were obtained, which suggests that other adhesion molecules also contribute to the adhesion of melanoma cells. This is supported by the lack of correlation between expression of MACAM-1 and the capacity of the various melanoma cells to adhere. Indeed, it has been reported that other melanoma-associated adhesion molecules are expressed on human melanoma cells (Vollkers and Birchmeier, 1983a,b; Dippold *et al.*, 1984; Cheresh *et al.*, 1985). Recently, gangliosides GD2 and GD3, which are strongly expressed on cells of neuroectodermal origin, were found to be associated with melanoma cell-substrate interaction (Cheresh *et al.*, 1985). Addition of an MAb directed against ganglioside GD3 to attached melanoma cells resulted in a rounding-up and detachment of these cells (Dippold *et al.*, 1984). The gangliosides GD2 and GD3 are not only major components of the plasma membrane, but are also deposited on adhesion plaques at the interface between the melanoma cells and the substratum to which these cells are attached (Cheresh *et al.*, 1985). Similar adhesion plaques containing high-MW proteo-

glycan and antigens detected by AMF-2 and AMF-7 were observed to be deposited on glass coverslips after melanoma cells were removed with EDTA. Class-I and Class-II MHC antigens, which are major membrane constituents of melanoma cells, and the melanoma-associated antigen detected by 96.5 (Hellström *et al.*, 1982) were absent from adhesion plaques. This indicates that MACAM-1, the high MW proteoglycan, and the antigen detected by AMF-2 are also components of the extracellular matrix produced by melanoma cells.

AMF-6 and AMF-7 were selected on the basis of their capacity to inhibit the reaction of cytotoxic T-lymphocyte (CTL) clones that preferentially lyse human melanoma cells (De Vries *et al.*, 1984). We have shown that this blocking reactivity was specific and have concluded that these CTL clones have receptors for the high-MW melanoma-associated proteoglycan and MACAM-1, which allows them to interact with human melanoma cells.

Our results indicate that MACAM-1, as detected by AMF-7 (and PAF-1), and the proteoglycan detected by AMF-6 are associated with biological functions of human melanoma cells that may play a role in the hematogenic dissemination of malignant melanoma. Identification of other MACAMs, and particularly their "counter-structures" on endothelial and other tissues, are relevant for gaining better insight into the process of metastasis in human melanoma.

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